Agrobacterium-Mediated Genetic Transformation of Switchgrass

M. N. Somleva, Z. Tomaszewski, and B. V. Conger*

ABSTRACT

Although *Agrobacterium tumefaciens* has been successfully used to transfer genes to a wide range of plant species, it has received little attention for transformation of forage grasses. Therefore, the objective of the present study was to demonstrate *Agrobacterium*-mediated transformation of switchgrass (*Panicum virgatum* L.). The *A. tumefaciens* strain AGL 1 carrying the binary vector pDM805, coding for the phosphinothricin acetyltransferase (*bar*) and β-glucuronidase (*gus*) genes, was utilized in these experiments. Somatic embryos, embryogenic calluses, mature caryopses, and plantlet segments served as target tissues for infection. Treated cultures were selected in the presence of 10 mg L^{-1} bialaphos and the resultant plantlets were treated with the herbicide Basta [monoammonium 2-amino-4(hydroxymethylphosphinyl)butanoate]. T-DNA delivery efficiency was affected by genotype, explant used and the presence or absence of acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) during inoculation and cocultivation. Approximately 600 transgenic plants were produced, and transformation efficiencies ranged from 0 to nearly 100%. Stable integration, expression, and inheritance of both transgenes were confirmed by molecular and genetic analyses. Approximately 90% of the tested plants appeared to have only one or two copies of the T-DNA inserts. Controlled crosses between T_0 and nontransgenic 'Alamo' plants indicated the expected ratio of 1:1 (transgenic:nontransgenic) in T_1 plants for both transgenes according to a χ^2 test at $P = 0.05$. These results indicate that the *Agrobacterium* method is effective for transferring foreign genes into switchgrass.

SWITCHGRASS is a warm season (C_4) perennial grass that is native to the tall grass prairies of North America (Moser and Vogel, 1995). Although switchgrass is an important forage crop, it has also recently received interest for its potential as a bioenergy crop (Sanderson et al., 1996; McLaughlin et al., 1999). Important to the improvement of this species is the development of cellular and molecular approaches, including gene transfer, that can be used to supplement conventional breeding programs.

The current status of forage and turf grass biotechnology has been recently reviewed (Forster and Spangenberg, 1999; Spangenberg et al., 2001; Wang et al., 2001). Transgenic plants have been reported for only 6 genera and 11 species (Wang et al., 2001); the only warm season grass listed is switchgrass. This was achieved in our laboratory by microprojectile bombardment of embryogenic calluses with a GFP-BAR plasmid (Richards et al., 2001). Integration of both the green fluorescent protein (*gfp*) and *bar* genes was shown by Southern blot hybridization. Fluorescing pollen was observed in T_0 plants and the *bar* gene was transmitted through both male and female gametes and expressed in T_1 progeny.

Microprojectile bombardment and gene transfer by *A. tumefaciens* are currently the two most common methods for achieving genetic transformation in higher plants. Although not universally accepted (Smith et al., 2001), it has been reported that *Agrobacterium*-mediated transformation leads to clean, discrete, low copy, well-defined, unrearranged DNA insertions into the plant genome (Chilton, 1993; Repellin et al., 2001; Upadhyaya et al., 2000). There are now several publications describing genetic transformation in various cereal species using *Agrobacterium* (Repellin et al., 2001). The only previous report of gene transfer in a forage or turf grass by this method was for GFP in creeping bentgrass {*Agrostis palustris* Huds. [= *A. stolonifera* var. *palustris* (Huds.) Farw.]; Yu et al., 2000}.

The objectives of the present study were to demonstrate high efficiency *Agrobacterium*-mediated transformation in switchgrass, and to show sexual transmission of the transgenes and their expression in T_1 progeny. The accomplishment of such provides an alternative to microprojectile bombardment for genetic manipulation of this species.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Embryogenic calluses were initiated from various explants of different genotypes of cv. Alamo according to established procedures (Table 1). Callus induction medium consisted of Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) supplemented with 22.5 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 μ*M* 6-benzylaminopurine (BAP). Maltose (30 g L^{-1}) was substituted for sucrose. Cultures were maintained at 27°C in the dark. Resulting calluses were subcultured every 4 wk. For plant regeneration, calluses were transferred to MS medium supplemented with 1.4 μ*M* gibberellic acid (GA_3) and incubated at 27°C with a 16-h photoperiod (cool white fluorescent bulbs at 80 μmol m^{-2} s^{-1}).

Agrobacterium Strain, Plasmid, and Culture

The transformation experiments were conducted using *A. tumefaciens* strain AGL1 (Lazo et al., 1991) containing the 18.15-kilobase (kb) transformation vector pDM805 (Tingay et al., 1997). This plasmid contains the *bar* gene under the control of the maize ubiquitin 1 (*Ubi*1) promoter and the *uid*A (*gus*) gene under the control of the rice actin 1 (*Act*1) promoter.

The *Agrobacterium* was grown from a single colony in MG/L medium (Garfinkel and Nester, 1980) supplemented

M.N. Somleva, USDA-ARS-PWA, Western Regional Research Center, 800 Buchanan St., Albany, CA 94710-1105; Z. Tomaszewski, Zabinskiego 18, Apt. 30, 02-793 Warsaw, Poland; B.V. Conger, Dep. of Plant Sciences and Landscape Systems, Univ. of Tennessee, 2431 Center Drive, Knoxville, TN 37996-4561. Received 28 Jan. 2001. *Corresponding author (congerbv@utk.edu).

Published in Crop Sci. 42:2080–2087 (2002).

Abbreviations: *Act*1, actin 1 promoter; *bar*, phosphinothricin acetyltransferase gene; GA_3, gibberellic acid; *gfp*, green fluorescent protein gene; *gus*, β-glucuronidase gene; kb, kilobase; MS, Murashige and Skoog medium; PCR, polymerase chain reaction; *Ubi*1, ubiquitin 1 promoter; X-Gluc, 5-bromo-4-chloro-3-indolyl-3-glucuronic acid.

with 20 mg L^{-1} rifampicin and 5 mg L^{-1} tetracycline at 27°C for 40 h. Aliquots of this culture (200 μL) were mixed with 200 μL of 15% aqueous glycerol and stored at −80°C. Bacteria were grown in 10 mL MG/L medium (with or without acetosyringone) at 27°C for 24 h and the resultant cultures (OD_{600} 0.560) were used for transformation.

Inoculation and Cocultivation

Approximately 25 immature somatic embryos collected from embryogenic callus or 10 to 15 callus pieces (2 by 2 mm each) were transferred into 1.5 mL of *A. tumefaciens* suspension in multiwell plates and incubated at 27°C for various periods (3–60 min) in the dark. After inoculation, the explants were transferred with a wide-mouth pipette into Petri dishes containing 25 mL of the MS medium described above. Cocultivation was performed at 27°C in the dark for 3 to 5 d.

Basal parts (5–6 mm) of plantlets, obtained from somatic embryos, were cut transversely into smaller pieces (2–3 mm) in the presence of *A. tumefaciens* and inoculated in the bacterial suspension at 27°C in the dark for 1 h. In some experiments, explants were precultured on callus induction medium for 5 or 10 d. Both uncultured and precultured segments were wounded with carborundum before inoculation. Ten segments were placed in a test tube containing 10 mg carborundum in 5 mL of liquid MS medium without plant growth regulators and vortexed at a low speed for 1 min. Cocultivation was performed on the induction medium at 27°C in the dark for 3 to 7 d.

Mature caryopses were dehusked in 60% H_2SO_4 and sterilized with commercial bleach as previously described (Denchev and Conger, 1994). Caryopses were infected with *A. tumefaciens* by incubation in the bacterial suspension at 27°C for 1 h in the dark followed by transfer to callus induction medium and coculture for 3 to 5 d. Caryopses were also precultured on callus induction medium for 2 wk. Explants with formed callus were infected with *Agrobacterium* using the procedure for embryogenic callus.

In all experiments, the virulent system of the bacterium was stimulated by addition of 50 or 200 μ*M* acetosyringone to the media for inoculation and/or cocultivation of somatic embryos and embryogenic callus. Mature caryopses and plantlet segments were infected with *Agrobacterium* cultures grown in the presence of 100 μ*M* acetosyringone.

β-Glucuronidase Activity Assay

β-Glucuronidase activity was assayed histochemically in explants and callus tissues after cocultivation as well as after 1 wk culture on the selection medium by incubation for a period of 16 h in a 5-bromo-4-chloro-3-indolyl-3-glucuronic acid (X-Gluc) solution as described by Jefferson et al. (1987). Twenty percent methanol was added to eliminate endogenous GUS activity (Kosugi et al., 1990). Explants stained for GUS activity immediately after cocultivation were incubated in a liquid MS medium supplemented with 150 mg L^{-1} Timentin overnight to eliminate the *Agrobacterium* before incubation in the X-Gluc solution. β-Glucuronidase expression was also examined in leaf tissue, tillers, and floral organs (anthers and ovaries).

Selection of Bialaphos-Resistant Callus Lines and Putative Transformants

Following cocultivation with *Agrobacterium*, the explants were transferred to callus induction medium containing 150 mg L^{-1} Timentin to eliminate the bacterium. Three different bialaphos selection schemes were evaluated. In the first method, tissues were cultured for 7 to 10 d without bialaphos before transfer to selection medium containing 10 mg L^{-1} bialaphos. In the second method, cocultured tissues were first placed on MS medium supplemented with 3 mg L^{-1} bialaphos and then transferred to MS medium containing 10 mg L^{-1} bialaphos. The third selection method involved transferring explants directly onto medium with 10 mg L^{-1} bialaphos immediately after cocultivation with the *Agrobacterium*. All explants were maintained on the medium containing 10 mg L^{-1} bialaphos for 4 wk with a biweekly subculture. During subculture, each piece of callus derived from one explant or one piece of inoculated callus was divided into several small pieces. Vigorously growing calluses were transferred to a regeneration medium (MS with 1.4 μ*M* GA_3) containing 10 mg L^{-1} bialaphos.

Table 1. Explant source for callus initiation from various switchgrass genotypes (our designation), cv. Alamo, used in the transformation experiments.

Alamo genotype	Initial explants	References
C10	Mature caryopses	Denchev and Conger, 1995
C21	Mature caryopses	
C50	Mature caryopses	
S01	Seedlings	Denchev and Conger, 1994
S07	Seedlings	
2	Immature inflorescences	Alexandrova et al., 1996
23	Immature inflorescences	
0108	Immature inflorescences	
2702	Immature inflorescences	

Resultant plantlets were tested for their response to Basta at different stages of growth by rubbing the leaves with sterile Q-tips soaked with a 0.1% solution of the herbicide. The first treatment was applied in vitro when the plantlets were growing on the selection medium and had 3 to 4 leaves. Two weeks after the treatment, the number of Basta-tolerant and Basta-sensitive plantlets obtained from each explant was determined and the percentage of the escapes was calculated. Plantlets showing no reaction to the herbicide were transferred to Magenta boxes (Sigma Chemical Co., St. Louis, MO) containing MS medium without the selection agent. They were again tested for their reaction to Basta at the transfer to 1-L polypropylene culture vessels (Phytacon, Sigma Chemical Co.) containing the same medium. After another 2 to 3 wk of culture, transformation frequency was determined as the number of tolerant plants recovered per explant inoculated. More than 100 plants were treated with Basta for the third time and transferred to moist peat pellets. Sixty plants were randomly selected, established in soil, and transferred to greenhouse benches. They received a final Basta treatment in the greenhouse by rubbing at least one leaf of each tiller with 0.1% of the herbicide.

Molecular Analyses

Genomic DNA of control, T_0, and T_1 plants was isolated from 250 mg of young leaf tissue using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Polymerase chain reaction (PCR) was used to screen 30 putative T_0 plants for presence of both the *gus* and *bar* genes. Primer sets used were: 5′-CAGGAAGTGATGGAGCATCAG-3′ and 5′-TCG TGCACCATCAGCACGTTA-3′ for *gus* (Becker et al., 1994), and 5′-CTGAAGTCCAGCTGCCAGAA-3′ and 5′-CATCG TCAACCACTACATCG-3′ for *bar* (Denchev et al., 1997). On the basis of this screening, 21 plants were selected for Southern blot hybridization. A PCR analysis to test for the presence of a conserved region of the *vir*C gene of *Agrobacterium* was also performed on these 21 plants using the primers 5′-ATGATTTGTAGCGGACT-3′ and 5′-AGCTCAACCTG

CTTC-3′ (Sawada et al., 1995). The predicted sizes of the amplified DNA fragments were 438 bp, 637 bp, and 730 bp for *bar*, *gus*, and *vir*C, respectively. The PCR conditions were as previously described (Denchev et al., 1997; Sawada et al., 1995).

Southern blot analyses were performed with 15 μg of DNA digested with the restriction enzymes *Bam*HI and *Spe*I in separate experiments. Digested DNAs were size-fractionated by agarose gel electrophoresis and transferred to a Hybond-N nylon membrane following the manufacturer's protocol (Amersham Pharmacia Biotech). The digoxigenin-labeled *bar* and *gus* probes for hybridization of *Bam*HI-digested DNA from primary transformants were generated by PCR amplification (PCR DIG Probe Synthesis Kit, Roche Molecular Biochemicals, Indianapolis, IN) according to Tingay et al. (1997). Membrane hybridization and post-hybridization washes were conducted as described by Engler-Blum et al. (1993). Detection of the digoxigenin-hybridized fragments was performed by enzyme immunoassay and enzyme-catalyzed color reaction (DIG Nucleic Acid Detection Kit, Roche Molecular Biochemicals) as specified by the manufacturer. For hybridization of *Spe*I-digested DNA, a 1.8-kb *Kpn*I-*Nco*I fragment representing the *gus* gene and a 0.54-kb *Kpn*I-*Pst*I fragment containing the *bar* gene isolated from the plasmid pDM805 were used as templates for the probe synthesis. The [^{32}P]-labeled probes were obtained by using the RadPrime DNA Labeling System (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions.

PCR analyses for presence of the *gus* and *bar* genes in T_1 progeny were performed as previously outlined for T_0 plants. To confirm that the amplified fragments corresponded to the two genes, the PCR gels were blotted for Southern analysis and hybridized with [^{32}P]-labeled probes as described above.

Progeny Analysis

Controlled crosses were made between transgenic plants, used as both male and female parents, and control Alamo plants. T_1 offspring were assayed for Basta tolerance by rubbing the seedling leaves with a 0.1% solution of the herbicide and for GUS by using the histochemical assay of leaf tissues. Data were analyzed by the χ^2 test at $P = 0.05$.

RESULTS

Transformation and Selection of Putative Transformants

Cutting of seedling segments in the presence of *A. tumefaciens* resulted in appearance of GUS expression only on the edges of the segments (Fig. 1A). Preculture of explants on callus induction medium for 5 or 10 d improved the frequency of GUS expression. The highest activity was observed after vortexing the segments with carborundum before inoculation (Fig. 1B). β-Glucuronidase gene expression was detected in the embryo region of mature caryopses after cocultivation with the bacterium (Fig. 1C). Embryogenic calluses initiated from various infected explants (Table 1) also expressed GUS (Fig. 1D).

Calluses after 2-wk culture on medium with 10 mg L^{-1} bialaphos are shown in Fig. 1E. Those that grew vigorously were divided into smaller pieces and transferred onto fresh selection medium for another 2 wk. At the end of this period, somatic embryos expressing GUS at different developmental stages were observed (Fig. 1F). Plantlets regenerated from resistant calluses and tested for their tolerance to Basta are shown in Fig. 1G. Tolerance to the herbicide indicated presence of the *bar* gene. More than 600 putative transgenic plantlets were produced across all experiments. Sixty randomly selected T_0 plants transferred to soil in a greenhouse are shown in Fig. 1H. All these plants were fertile and produced seeds after controlled crosses. T_0 plants also expressed GUS in pollen grains (Fig 1I), young ovaries (Fig. 1J), leaf tissues (Fig. 1K) and young tillers (Fig. 1L). Some plants that exhibited weak GUS activity in leaf tissues showed high GUS activity in floral parts and vice versa. Approximately 15% of these sixty T_0 plants showed GUS expression in leaf tissue samples within 6 h of adding X-Gluc substrate, while GUS activity was detected in 75% of the primary transformants after 16 h of incubation. No GUS expression was detected in 10% of the Basta-tolerant transformants. Results showed that coexpression of the *gus* and *bar* genes was 92% (55/60).

Effect of Acetosyringone on Transformation Efficiency

Acetosyringone did not affect the frequency of GUS expression when added to the media for inoculation and/or cocultivation. However, the presence of this compound increased the recovery of transgenic plants from most of the explants used. Data for the effect of acetosyringone on transformation efficiency of somatic embryos and established embryogenic calluses selected by the three methods described are summarized in Tables 2, 3, and 4. The highest transformation efficiency in Alamo genotype C50 was obtained when somatic embryos were both inoculated and cocultivated in the presence of 200 μ*M* acetosyringone (Table 2). Some of the somatic embryos transformed without acetosyringone also formed calluses during subsequent selection, but none produced transgenic plantlets. The highest number of Basta-tolerant plantlets produced from embryogenic calluses was obtained using media without acetosyringone either during inoculation or cocultivation. When 200 μ*M* acetosyringone was added to the inoculation medium, overgrowth of the bacterium was observed during the selection, which resulted in inhibition of callus growth. Data for transformation efficiency of somatic embryos and established callus cultures from other Alamo genotypes are shown in Tables 3 and 4, respectively. A total of 2550 calluses and 830 somatic embryos were used in the experiments. Only genotypes that produced at least one transgenic plant are included in the data presented. Depending on genotype, explant used, and acetosyringone concentration, transformation efficiencies ranged from 0 to nearly 100%. In general, transformation efficiency was higher with somatic embryos than with calluses.

Molecular Analyses of T_0 Plants

Polymerase chain reaction screening showed the presence of both transgenes in T_0 plants (data not shown). Southern blot analyses of 10 of these plants are shown in Fig. 2. All bands detected by hybridization with the *gus* probe represented fragments of >5 kb (Fig. 2A). The banding pattern observed demonstrated that most

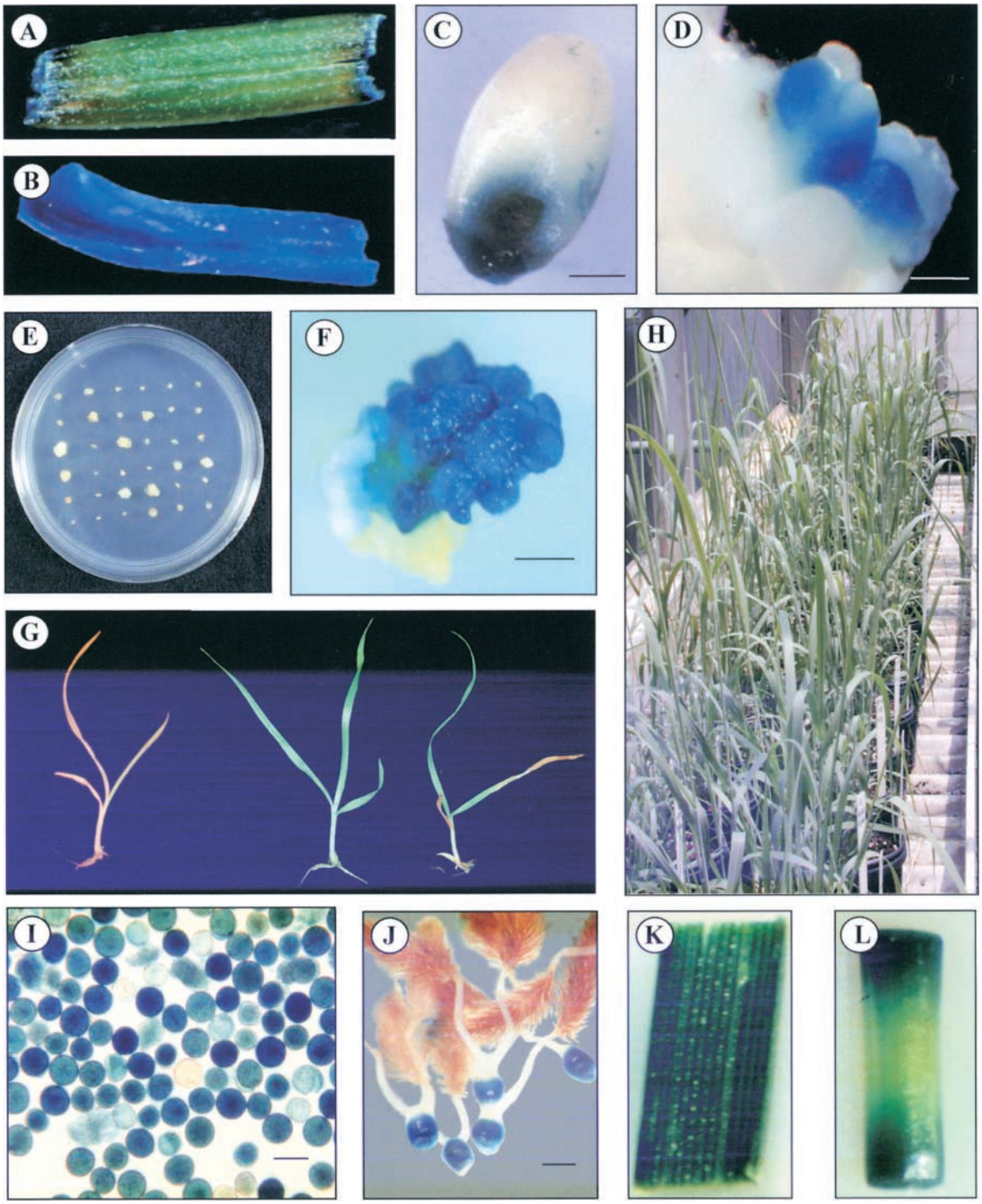

Fig. 1. ***Agrobacterium*-mediated transformation of various switchgrass explants and selection of putative transformants. (A) Transient GUS expression in a plantlet segment cut in the presence of *Agrobacterium*. (B) A segment wounded with carborundum before inoculation. (C) A mature caryopsis after cocultivation with *Agrobacterium*. (D) Transformed embryogenic callus. (E) Selection of calluses produced from inoculated somatic embryos subjected to selection with 10 mg L^{-1} bialaphos after cocultivation. (F) Callus with somatic embryos expressing GUS after selection on medium containing bialaphos for 5 wk. Cultures were initiated from mature caryopses and maintained for three months before infection with the *Agrobacterium*. (G) Response of plantlets regenerated on a selection medium to Basta after rubbing their leaves with 0.1% herbicide solution (left control, center, and right putative transformants). (H) Primary transformants in a greenhouse. (I–L) Stable GUS expression in floral and vegetative parts of T_0 plants: (I) pollen, (J) young ovaries, (K) leaf tissue, and (L) young tillers. Bar: 500 μm (C, D, and F); 50 μm (I); 300 μm (J).**

of the tested plants contained one or two gene copies. Only one plant (Lane 7) contained a large number of copies (at least eight). The membrane was stripped and probed again with the *bar* probe. One 3.7-kb band was detected, indicating that the *bar* gene was transferred to all tested plants (Fig. 2B). The higher intensity of the

Table 2. Effect of acetosyringone (AS) on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos and embryogenic calluses from Alamo genotype C50. E, number of inoculated explants; C, number of bialaphos-resistant calluses derived from them; P, number of Basta-tolerant plantlets; P/E, transformation efficiency.

Type of inoculated explants	AS concentration during inoculation	AS concentration during cocultivation, μ*M*											
		0				50				200			
		E	C	P	P/E	E	C	P	P/E	E	C	P	P/E
	μ*M*				%				%				%
Somatic embryos	0	54	32	0	0	20	6	0	0	24	5	8	33.3
	50	40	5	0	0	57	37	49	86.0	24	8	3	12.5
	200	50	8	0	0	24	6	0	0	74	41	72	97.3
Calluses	0	108	41	69	63.9	61	27	0	0	51	28	8	15.7
	50	108	29	11	10.2	131	48	10	7.6	59	31	6	10.2
	200	49	42	0	0	19	27	0	0	117	67	5	4.3

Table 3. Effect of acetosyringone (AS) on the transformation efficiency of somatic embryos from various Alamo genotypes. E, number of inoculated explants; C, number of bialaphos-resistant calluses derived from them; P, number of Basta-tolerant plantlets; P/E, transformation efficiency.

Alamo genotype	AS concentration during inoculation and cocultivation, μ*M*											
	0				50				200			
	E	C	P	P/E	E	C	P	P/E	E	C	P	P/E
				%				%				%
C10	28	3	0	0	32	4	8	25.0	39	13	33	84.6
C21	40	0	0	0	38	4	3	7.9	34	3	9	26.5
2	23	2	0	0	60	7	0	0	61	11	9	14.8
0108	32	0	0	0	23	6	6	26.1	34	10	0	0

Table 4. Effect of acetosyringone (AS) on the transformation efficiency of embryogenic calluses from various Alamo genotypes. E, number of inoculated explants; C, number of bialaphos-resistant calluses derived from them; P, number of Basta-tolerant plantlets; P/E, transformation efficiency.

Alamo genotype	AS concentration during inoculation	AS concentration during inoculation and cocultivation, μ*M*											
		0				50				200			
		E	C	P	P/E	E	C	P	P/E	E	C	P	P/E
	μ*M*				%				%				%
C10	0	55	14	0	0	62	30	55	88.7	54	26	21	38.9
	200	72	29	0	0	75	34	8	10.7	58	22	18	31.0
C21	0	46	10	0	0	49	13	19	38.8	50	13	48	96.0
	200	39	13	0	0	56	18	34	60.7	56	14	20	35.7
2702	0	57	18	0	0	60	29	10	16.7	48	18	0	0
	200	61	21	0	0	82	37	11	13.4	50	15	15	53.6
2	0	45	6	12	26.7	58	8	0	0	78	17	0	0
	200	55	8	0	0	54	4	0	0	53	6	0	0
S07	0	93	16	0	0	43	7	0	0	66	16	0	0
	200	54	8	0	0	46	14	1	2.2	50	12	0	0

signal in Lane 7 suggests the presence of multiple copies of the T-DNA. This is the same plant that showed numerous bands for *gus*.

To further confirm integration into the host genome, Southern blot analysis was performed with genomic DNA digested with *Spe*I. This enzyme cleaves the T-DNA at a unique restriction site near the right border. The [^{32}P]-labeled *gus* gene probe hybridized to bands >9 kb, which indicated that the whole T-DNA was inserted into the genome of the analyzed plants (Fig. 3). It is evident that the majority of plants have one or two copies of complex T-DNA inserts. PCR analysis conducted on 21 plants for presence of the *Agrobacterium Vir*C gene was negative.

Analysis of Progeny (T_1) Plants

Data for progeny from reciprocal crosses between transgenic and control plants are presented in Table 5. A segregation ratio of 1:1 among T_1 plants was obtained from all crosses. The transgenic plants used in the crosses had one or two insert copies as shown by DNA gel blot hybridization (Lanes 1, 4, 5, and 9 in Fig. 3). The presence of the transgenes in the offspring was confirmed by PCR analysis (Figs. 4A, B) and Southern hybridization of the PCR gels (Fig. 4C, D).

DISCUSSION

Although *Agrobacterium*-mediated transformation was used to produce transgenic plants of creeping bentgrass, a cool-season (C_3) turf grass (Yu et al., 2000), switchgrass is the first forage grass, cool or warm season, in which gene transfer by this method has been demonstrated. Factors, including genotype, type of tissue used for infection, preculture of explants, wounding of tissue prior to infection, acetosyringone during infection and cocultivation, and different methods of selection influenced transformation effectiveness. Generally, these are the same factors as reported for influencing *Agrobacterium*-

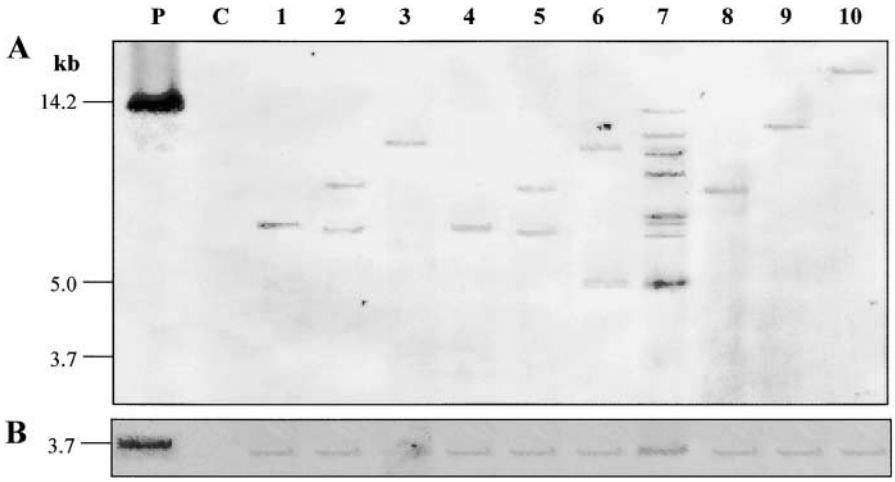

Fig. 2. Southern blot analysis of individual Basta-tolerant T_0 switchgrass plants. Genomic and plasmid DNA was digested with *Bam*HI. DIG dUTP was incorporated into the amplified PCR products of 326 bp (*gus*) and 357 bp (*bar*). (A) Blot of genomic DNA showing hybridization with the *gus* probe. (B) The same blot showing hybridization with the *bar* probe after stripping. Lane: P, plasmid pDM805; C, a control Alamo plant; 1–10, transgenic plants.

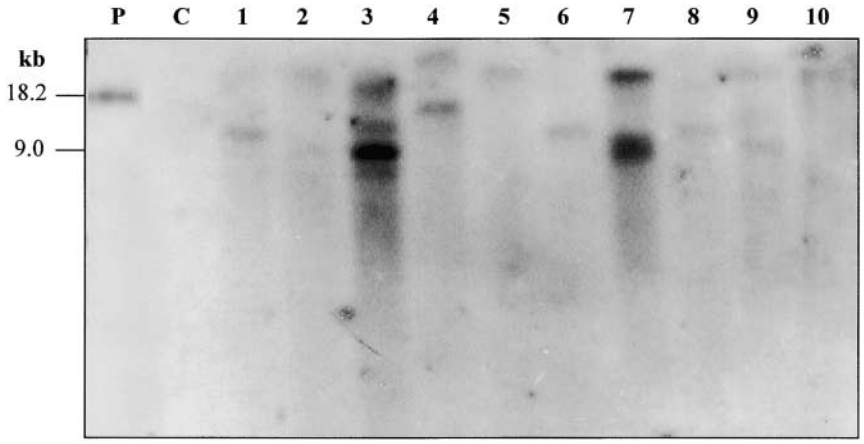

Fig. 3. Blot of *Spe*I-digested DNA from T_0 switchgrass plants presenting different copy numbers of the T-DNA after hybridization with the [^{32}P]-labeled *gus* probe. Lane: P, plasmid pDM805; C, a control Alamo plant; 1–10, transgenic plants. Sizes of the plasmid pDM805 (18.2 kb) and the T-DNA (9 kb) are also indicated.

mediated transformation in cereal species, such as wheat (*Triticum aestivum* L.; Weir et al., 2001), rice, (*Oryza sativa* L.; Hiei et al., 1994) and maize (*Zea mays* L.; Lupotto et al., 1999).

The highest efficiency for recovery of transgenic plants was obtained when embryogenic calluses and somatic embryos were used as targets for infection. Of these, somatic embryos were superior because they proliferated highly embryogenic callus, each of which produced numerous transformed plants during the selection process. Also, most of the recovered plantlets were transgenic, whereas ≈30% of the plantlets obtained from infected calluses were untransformed escapes.

Acetosyringone increased the frequency of transgenic plants recovered, especially from somatic embryos, and improved the efficiency in most of the genotypes utilized. These results are in agreement with most reports of *Agrobacterium*-mediated transformation of Poaceae species. Our obtainment of transgenic plants without acetosyringone is in disagreement with Azhakanandam et al. (2000), who reported that it was not possible to induce transient GUS expression in rice embryogenic callus in the absence of this compound. Our transformation efficiencies are among the highest reported for the *Agrobacterium* method in grass and cereal species and are higher than the 10 to 31% reported by Akhakanandam et al. (2000) for rice. The success is probably due to the virulence of the *Agrobacterium* strain (AGL 1) used in our experiments and the susceptibility of the target tissues. Also, application of Basta to young plantlets allowed early elimination of most of those that were untransformed. Only a few escapes were identified after the second herbicide treatment.

Another significant factor contributing to the success is the promoters in pDM805 driving transgene expression. The maize *Ubi*1 promoter is used for the *bar* gene and the rice actin (*Act*1) promoter for the *gus* gene. These are the same promoters that are in the GFP-BAR plasmid used in our previous switchgrass transformation with microprojectile bombardment (Richards et al. 2001). Generally, in most monocot systems, *Ubi*1 has proved to be the strongest promoter followed by *Act*1 (Li et al., 1997; McElroy and Brettell, 1994).

Although the genotypes used were all within the culti-

Table 5. Segregation analysis of T_1 plants derived from reciprocal crosses between T_0 and control (nontransgenic) 'Alamo' plants. The χ^2 test is based on expected segregation ratio of 1:1.

Cross	No. T_1 plants	T_1 plants assayed for Basta tolerance		Ratio T:S	χ^2	T_1 plants assayed for GUS activity		Ratio P:N	χ^2
		Tolerant (T)	Sensitive (S)			Positive (P)	Negative (N)		
3025 × $T_0$7	51	30	21	1:0.7	1.59	31	20	1:0.6	2.37
$T_0$7 × 3025	63	31	32	1:1	0.02	35	28	1:0.8	0.78
3125 × $T_0$35	110	60	50	1:0.8	0.91	59	51	1:0.9	0.58
$T_0$35 × 3125	60	25	35	1:1.4	1.67	28	32	1:1.1	0.27
2017 × $T_0$17	18	8	10	1:1.2	0.22	10	8	1:0.8	0.22
$T_0$17 × 2017	71	33	38	1:1.2	0.35	29	42	1:1.4	2.38
3125 × $T_0$8	70	37	33	1:0.9	0.23	38	32	1:0.8	0.51
$T_0$8 × 3125	89	39	50	1:1.3	1.36	44	45	1:0.9	0.01
2702 × $T_0$8	35	18	17	1:0.9	0.03	15	20	1:1.3	0.71
$T_0$8 × 2702	62	33	29	1:0.9	0.26	28	34	1:1.2	0.58

var Alamo, there were differences for recovering transgenic plants. Genotype differences for *Agrobacterium*-mediated transformation frequencies have also been reported for various Poaceae species, for example, different rice cultivars (Azhakanandam et al., 2000; Ke et al., 2001) and maize inbred lines (Lupotto et al., 1999).

Inoculation in the bacterial suspension for 3 to 60 min did not cause significant changes in the frequency of transgenic plants recovered. However, longer inoculation periods resulted in overgrowth of the *Agrobacterium*. Optimum cocultivation periods depended on target tissue used and were shorter for somatic embryos than for other explants. Transfer of infected explants to medium with low or no bialaphos to favor multiplication of transformed cells resulted in more vigorously growing calluses, but did not enhance transformation efficiency. Rather, it allowed for more untransformed escapes.

Southern blot hybridization after digesting the genomic DNA with *Bam*HI showed different hybridization patterns among all of the T_0 plants tested, indicating that T-DNA was integrated into the plant genome. The results obtained with *Spe*I, since this enzyme cuts the plasmid at only one site, provides further and even stronger evidence of transgene integration into the host genome. On the basis of the DNA gel blot hybridizations, ≈60% of the analyzed plants had single inserts, which is close to the 60 to 70% observed in maize (Ishida et al., 1996) and significantly greater than the 32% for rice (Hiei et al., 1994) and the 35% for wheat (Cheng et al., 1997). In the *Agrobacterium*-mediated transformation study with creeping bentgrass (Yu et al., 2000), two plants derived from the same callus showed two identical insertions and two other plants from a different callus showed only one insertion.

The high proportion of discrete integration events containing single or low T-DNA copy numbers is considered a desirable characteristic of *Agrobacterium* for gene transfer. However, multiple copies have been reported in transgenic plants of various plant species using this method (Smith et al., 2001). Only two T_0 switchgrass plants showed multiple insertions (3–8). The difference in the number of the bands detected in DNA from T_0 Plant 14 after digestion with *Bam*HI and *Spe*I suggests a rearrangement of some of the inserts, which often occurs in primary transformants containing multiple insert copies. The very weak GUS activity observed in leaf tissues and pollen of this plant suggests the possibility of gene silencing. High copy numbers also resulted in weak GFP expression in pollen of plants in our previous switchgrass transformation study (Richards et al., 2001). The other plants tested, which contained only one to two gene copies, showed stronger GUS expression.

Although progeny analyses have been reported for various transformation experiments with major cereals, there are only two reports of such with forage or turf grass species, switchgrass (Richards et al., 2001), and ryegrass (*Lolium* spp.) (Wang et al., 1997). This is probably because most of these grasses are self-incompatible and controlled pollinations must be made under controlled conditions. Although not stated, it appeared that the crosses made with ryegrass utilized the transgenic plants only as female parents; therefore, transmission of the transgenes through the male gametes was not demonstrated. The segregation ratios in the offspring obtained from crosses in the present study, showed the expected 1:1 for both traits when the transgenic plants were used as either the male or female parent. These data, combined with the PCR analyses of progeny, pro-

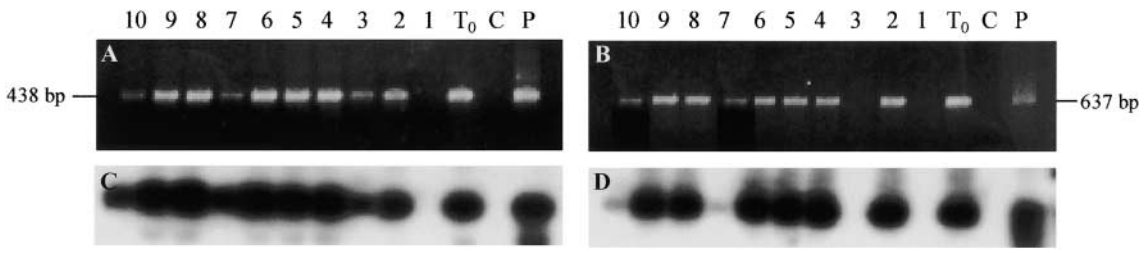

Fig. 4. Polymerase chain reaction (PCR) analysis of T_1 switchgrass progeny showing the 438 and 637 bp fragments corresponding to the *bar* (A) and *gus* (B) genes. Southern blot analysis of the PCR gels using [^{32}P]-labeled probes for the *bar* (C) and *gus* (D) genes. Lane: P, plasmid pDM805; C, a control Alamo plant; T_0, the female parent plant 35; 1, a T_1 plant which showed no transgene expression; 2–10, Basta-tolerant T_1 plants.

vide further confirmation for stable genetic transformation in these experiments.

In conclusion, *Agrobacterium*-mediated transformation has been demonstrated for switchgrass. Furthermore, the transgenes were sexually transmitted through both male and female gametes and expressed in T_1 progeny. Tolerance to the herbicide Basta represents transfer of a gene of practical agronomic importance. Basta is sold commercially under the name *Liberty*, and *Liberty Link* products are of high current interest because they represent an alternative to *Roundup Ready* as crops tolerant to a nonselective herbicide.

ACKNOWLEDGMENTS

We gratefully acknowledge supply of the *Agrobacterium* strain AGL 1 (pDM805) by Dr. Richard Brettell, CSIRO Division of Plant Industry, Canberra, Australia. Research was supported by the Bioenergy Feedstock Development Program of the U.S. Department of Energy under Contract No. 11X-SY 161C with the University of Tennessee-Battelle, LLC.

REFERENCES

Alexandrova, K.S., P.D. Denchev, and B.V. Conger. 1996. In vitro development of inflorescences from switchgrass nodal segments. Crop Sci. 36:175–178.

Azhakanandam, K., M.S. McCabe, J.B. Power, K.C. Lowe, E.C. Cocking, and M.R. Davey. 2000. T-DNA transfer, integration, expression and inheritance in rice: effects of plant genotype and *Agrobacterium* super-virulence. J. Plant Physiol. 157:429–439.

Becker, D., R. Brettschneider, and H. Lörz. 1994. Fertile transgenic wheat from microprojectile bombardment of scuttelar tissue. Plant J. 2:299–307.

Cheng, M., J.E. Fry, S. Pang, H. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Connor, and Y. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115:971–980.

Chilton, M. 1993. *Agrobacterium* gene transfer: Progress on a "poor man's vector" for maize. Proc. Nat. Acad. Sci. USA 90:3119–3120.

Denchev, P.D., and B.V. Conger. 1994. Plant regeneration from callus cultures of switchgrass. Crop Sci. 34:1623–1627.

Denchev, P.D., and B.V. Conger. 1995. *In vitro* culture of switchgrass: Influence of 2,4-D and picloram in combination with benzyladenine on callus initiation and regeneration. Plant Cell Tissue Organ Cult. 40:43–48.

Denchev, P.D., D.D. Songstad, J.K. McDaniel, and B.V. Conger. 1997. Transgenic orchardgrass (*Dactylis glomerata* L.) plants by direct embryogenesis from microprojectile bombarded leaf cells. Plant Cell Rep. 16:813–819.

Engler-Blum, G., M. Meier, J. Frank, and G.A. Muller. 1993. Reduction of background problems in nonradioactive Northern and Southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. Anal. Biochem. 210:235–244.

Forster, J.W., and G.C. Spangenberg. 1999. Forage and turfgrass biotechnology, principles, methods, and prospects. p. 191–237. *In* J.K. Setlow (ed.) Genetic engineering. Kluwer Academic/Plenum, New York.

Garfinkel, M., and E.W. Nester. 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumourigenesis and octopine catabolism. J. Bacteriol. 144:732–743.

Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271–282.

Ishida,Y., H. Saito, S. Ohta, Y. Hiei, T. Komari, and T. Kumashiro. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14:745–750.

Jefferson, R.A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907.

Ke, J., R. Khan, T. Johnson, D.A. Somers, and A. Das. 2001. High-efficiency gene transfer to recalcitrant plants by *Agrobacterium tumefaciens*. Plant Cell Rep. 20:150–156.

Kosugi, S., Y. Ohashi, K. Nakajima, and Y. Arai. 1990. An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. 70:133–140.

Lazo, G.R., P.A. Stein, and R.A. Ludwig. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio/Technology 9:963–967.

Li, Z., N.M. Upadhyaya, S. Meena, A.J. Gibbs, and P.M. Waterhouse. 1997. Comparison of promoters and selectable marker genes of use in Indica rice transformation. Mol. Breed. 3:1–14.

Lupotto, E., A. Reali, S. Passera, and M.-T. Chan. 1999. Maize elite inbred lines are susceptible to *Agrobacterium tumefaciens*-mediated transformation. Maydica 44:211–218.

McElroy, D., and R. Brettel. 1994. Foreign gene expression in transgenic cereals. Trends Biotechnol. 12:62–68.

McLaughlin, S.B., J. Bouton, D. Bransby, B.V. Conger, W.R. Ocumpaugh, D. Parrish, C. Taliaferro, K. Vogel, and S. Wullschleger. 1999. Developing switchgrass as a bioenergy crop. p. 282–299. *In* J.J. Janick (ed.) Perspectives on new crops and new uses. Am. Soc. Hortic. Sci. Press, Alexandria, VA.

Moser, L.E., and K.P. Vogel. 1995. Switchgrass, big bluestem, and indiangrass. p. 409–420. *In* R.F. Barnes et al. (ed.) Forages. Vol. I: An introduction to grassland agriculture. Iowa State Univ. Press, Ames, IA.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Repellin, A., M. Båga, P.P. Jauhar, and R.N. Chibbar. 2001. Genetic enrichment of cereal crops via alien gene transfer: New challenges. Plant Cell Tissue Organ Cult. 64:159–183.

Richards, H.A., V.A. Rudas, H. Sun, J.K. McDaniel, Z. Tomaszewski, and B.V. Conger. 2001. Construction of a GFP-BAR plasmid and its use for switchgrass transformation. Plant Cell Rep. 20:48–54.

Sanderson, M.A., R.L. Reed, S.B. McLaughlin, S.D. Wullschleger, B.V. Conger, D.J. Parrish, D.D. Wolf, C. Taliaferro, A.A. Hopkins, W.R. Osumpaugh, M.A. Hussey, J.C. Read, and C.R. Tischler. 1996. Switchgrass as a sustainable bioenergy source. Bioresour. Technol. 56:83–93.

Sawada, H., H. Ieki, and I. Matsuda. 1995. PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol. 61:828–831.

Smith, N., J.B. Kilpatrick, and G.C. Whitelam. 2001. Superfluous transgene integration in plants. Crit. Rev. Plant Sci. 20:215–249.

Spangenberg, G., R. Kalla, A. Lidgett, T. Sawbridge, E.K. Ong, and U. John. 2001. Transgenesis and genomics in molecular breeding of forage plants. p. 615–625. *In* J.A. Gomide et al. (ed.) Proc. Int. Grassland Congress, 19th, São Pedro, São Paulo, Brazil. 11–21 Feb. 2001. Fundação de Estudos Agrários Luiz de Queiroz, Piracicaba, Brazil.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton, and R. Brettell. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11:1369–1376.

Upadhyaya, N.M., B. Surin, K. Ramm, J. Gaudron, P.H.D. Schunmann, W. Taylor, P.M. Waterhouse, and M.B. Wang. 2000. *Agrobacterium*-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. Aust. J. Plant Physiol. 27:201–210.

Wang, G.R., H. Binding, and U.K. Posselt. 1997. Fertile transgenic plants from direct gene transfer to protoplasts of *Lolium perenne* L. and *Lolium multiflorum* Lam. J. Plant Physiol. 151:83–90.

Wang, Z., A. Hopkins, and R. Mian. 2001. Forage and turf grass biotechnology. Crit. Rev. Plant Sci. 20:573–619.

Weir, B., M.B. Wang, N. Upadhyaya, A.R. Elliott, and R.I.S. Brettell. 2001. *Agrobacterium tumefaciens*-mediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker. Aust. J. Plant Physiol. 28:807–818.

Yu, T.T., Z. Skinner, G.H. Liang, H.N. Trick, B. Huang, and S. Muthukrishnan. 2000. *Agrobacterium*-mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas 133:229–233.